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The Equilibrium Constants of the Glutamate Dehydrogenase Systems

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1. Equilibrium constants for the oxidation of glutamate by NAD^+ and $NADP^+$, catalysed by glutamate dehydrogenase, have been measured in phosphate buffers of different ionic strengths and at several temperatures. 2. The equilibrium constants for both systems vary markedly with ionic strength. Thermodynamic values for the two systems obtained by extrapolation to zero ionic strength differ significantly from one another. The standard free-energy change for $NADP^+$ reduction has been calculated from that for NAD^+ reduction. 3. The heat of reaction has been estimated and is the same with both coenzymes. 4. The thermodynamic data are discussed in relation to earlier data.

Equilibrium constants or free-energy changes for enzyme-catalysed reactions are fundamental biochemical constants. Accurate estimates of these quantities are important for studies of the mechanisms of biological energy transformations (cf. Burton & Krebs, 1953) and, in conjunction with initial-rate parameters, of the mechanisms of individual enzyme-catalysed reactions (Alberty, 1953). Liver glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase, EC 1.4.1.3] is one of the few dehydrogenases that exhibits comparable activity with both nicotinamide nucleotide coenzymes. Equilibrium constants for both systems have been measured previously, but only at high ionic strength and at 27° (Olsen & Anfinsen, 1953). From these data, together with an independent value for the standard free energy of reduction of NAD^+ at low ionic strength, the standard free-energy change for $NADP^+$ reduction was calculated (Burton & Wilson, 1953). The result was recognized as approximate, as it depends on the assumption that the ratio of the two equilibrium constants does not vary with ionic strength. Studies of the equilibrium constants of the glutamate dehydrogenase reactions over a range of ionic strengths were needed to obtain a more reliable value.

Glutamate dehydrogenase is present in liver in high activity and entirely in the mitochondrial matrix (Delbrück, Schimassek, Bartsch & Bücher, 1959). It has therefore been assumed that this enzyme effectively establishes equilibrium *in vivo* between its substrates and the free nucleotides, and that direct measurements of the tissue content of the substrates, together with a knowledge of the equilibrium constant under physiological conditions, would allow estimation of the NAD^+/NADH concentration ratio in the liver mitochondria (Williamson, Lund & Krebs, 1967).

The objective of the work described in the present paper was to determine the equilibrium constants for the glutamate dehydrogenase systems over wide ranges of ionic strength and temperature, including physiological values. These data were also needed for kinetic studies of the mechanism of this enzyme.

MATERIALS AND METHODS

Enzyme. Crystalline ox liver glutamate dehydrogenase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The suspension of crystals in $(NH_4)_2SO_4$ solution was centrifuged at 2°, and the enzyme dissolved in 0·2 M-sodium phosphate buffer, pH 7·0, containing EDTA (10 μM) and thoroughly dialysed against the same buffer, in which it is quite stable.

Coenzymes. NAD^+ and $NADP^+$ were from C. F. Boehringer und Soehne G.m.b.H., and the 3-acetylpyridine analogue of NAD^+ was from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. NAD^+ was assayed with alcohol dehydrogenase (Dalziel, 1961) and $NADP^+$ with glutamate dehydrogenase. For the $NADP^+$ assay the reaction mixture was 1 ml. of 0·45 M-L-glutamate, 2 ml. of 0·4 M-phosphate buffer, pH 7·9, 0·1 ml. of $NADP^+$ solution (1 mg./ml.) and 0·1 ml. of glutamate dehydrogenase solution (2 mg./ml.). Reduction of $NADP^+$ was complete (99·95%) in 10 min. With greater glutamate concentrations, or higher pH, the reaction was slower. This method gave results in good agreement with those obtained by the assay with glucose 6-phosphate dehydrogenase described by Horecker & Kornberg (1957).

Substrate. The monosodium salt of L-glutamic acid was obtained as the monohydrate from Frederick Boehm Ltd., London, W. 1, and from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Assays with glutamate dehydrogenase and oxidized acetylpyridine–adenine dinucleotide (Holzer, Söling & Witt, 1963) gave values within 1% of the theoretical values for both preparations.

Buffer solutions. Phosphate buffer solutions of various ionic strengths in the range I 0·02–0·70 at pH 6·9–7·1 were

prepared by mixing stock solutions of NaH_2PO_4 and Na_2HPO_4 in proportions estimated from the data of Cohn tabulated by Green & Hughes (1955). The stock phosphate solutions, 0·2–0·6 M, were freshly prepared for each experiment from the dry anhydrous salts of A.R. grade. The $NaH_2PO_4,2H_2O$ was heated for 2 hr. at 100°, and Na_2HPO_4 was dried by heating for 2 hr. at 110°.

The ionic strengths of the reaction mixtures with respect to phosphate buffer were calculated from the equation:

$$I = 0{\cdot}5\Sigma c_i z_i^2$$

where c_i is the molarity of an ion and z_i its valency. The small contribution by glutamate to the ionic strength was calculated from the same formula on the assumption that the net charge of the glutamate^{+2-} ion is unity. As the concentrations of NAD^+ and $NADP^+$ were at most 0·5 mM, and those of NADH, NADPH, α-oxoglutarate and NH_4^+ at equilibrium no more than 0·1 mM, the contributions of these species to the ionic strength were considered to be negligible.

Measurements of pH. The pH of equilibrium mixtures was measured at the temperature of the experiment in a water-jacketed electrode vessel. Either a direct-reading pH-meter (Electronic Instruments Ltd., model 23A) or a Vibron electrometer with pH-measuring attachment (Electronic Instruments Ltd., model C33B) was used with the recommended glass and calomel electrodes. The instruments were standardized with a buffer solution of 25 mM-KH_2PO_4–25 mM-Na_2HPO_4, pH 6·865, at 25° (Bates, 1964). The accuracy of the pH measurements was estimated as ±0·005 pH unit and this is probably the major source of error in estimates of the equilibrium constant.

Equilibrium measurements. Relatively large enzyme concentrations, up to 0·1 mg./ml., were added to reaction mixtures of NAD^+ or $NADP^+$ and glutamate in phosphate buffer containing 10 μM-EDTA to reach equilibrium quickly (10–15 min.) and to minimize coenzyme breakdown (Winer

Table 1. *Glutamate dehydrogenase equilibrium with NAD^+ in phosphate buffers of various ionic strengths at 27°*

Concentrations of NAD^+ and glutamate are the initial values and those of NADH are the equilibrium values. The numbers of equilibrium mixtures measured in each experiment are given in parentheses in Column (7). Values of K_c are calculated from eq. (1).

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
			[NAD^+]	[Glutamate]	[NADH]	$10^{15}K_c$	$10^{15}K_c$ (av.)
I	Expt. no.	pH	(μM)	(mM)	(μM)	(M)	(M)
0·0245	8	6·83	501	1·35	62	1·13 (2)	1·01
	9	6·93	508	1·35	62	0·88 (3)	
0·0445	8	6·82	501	1·35	69	1·59 (2)	1·39
	9	6·94	508	1·35	69	1·19 (3)	
0·055	3	6·90	165	2·0–3·3	52–61	1·45 (4)	1·35
	5	6·90	102	2·0–3·3	40–48	1·28 (6)	
	6	7·07	154	2·0–3·3	55–64	1·33 (6)	
0·067	7	6·92	506	1·34	73	1·53 (3)	1·49
	8	6·92	501	1·35	73	1·55 (2)	
	9	6·94	508	1·35	72	1·41 (3)	
	12	6·89	483	2·26	85	1·47 (3)	
0·089	7	6·98	506	1·34	78	1·64 (3)	1·71
	8	6·93	501	1·35	78	1·87 (2)	
	9	6·95	508	1·35	77	1·58 (3)	
0·101	3	6·90	165	2·0–3·3	55–63	1·71 (3)	1·77
	6	7·06	154	2·0–3·3	60–67	1·82 (6)	
0·111	7	6·97	506	1·34	81	1·91 (3)	1·88
	8	6·98	501	1·35	82	1·92 (2)	
	9	6·96	508	1·35	79	1·81 (3)	
0·135	8	6·98	501	1·35	83	2·05 (2)	2·06
	9	6·95	508	1·35	82	2·10 (3)	
	12	6·97	201	2·26	68	2·04 (4)	
0·233	1	6·95	164	0·74–2·95	38–43	2·63 (6)	2·68
	3	6·90	165	3·7–7·4	71–88	2·83 (4)	
	12	6·99	483	2·26	105	2·58 (3)	
0·471	1	6·90	164	0·6–3·3	46– 75	3·45 (9)	3·40
	2	6·90	167	3·3–22·2	75–118	3·39 (6)	
	2	7·17	167	2·6–3·3	70– 90	3·40 (3)	
	3	6·90	165	3·3–6·6	76– 89	3·44 (3)	
	10	6·79	491	2·3	99	3·31 (3)	
	11	6·99	489	2·3	114	3·41 (3)	
	12	6·99	204	2·3	78	3·38 (4)	
0·709	3	6·89	165	7·4–14·7	90–108	3·72 (4)	3·74
	12	7·02	483	2·3	118	3·76 (3)	

& Schwert, 1958). No significant variation of the equilibrium constant with enzyme concentration was observed. The reaction mixtures were made up in test tubes and incubated in a water bath at the required temperature, and transferred to quartz cells, optical path 1 cm., for extinction measurements at 340 mμ in a Zeiss spectrophotometer, model PMQII, equipped with a thermostatically controlled cell housing. Blank measurements were made with reaction mixtures from which coenzyme was omitted. The concentration of reduced coenzyme at equilibrium was calculated from the extinction coefficient of $6{\cdot}22 \times 10^6$ cm.^{2}/mole (Horecker & Kornberg, 1948). The equilibrium constant, K_c, was calculated from the equation:

$$K_c = \frac{[\text{NAD(P)H}][\text{2-oxoglutarate}^{2-}][\text{NH}_4^+][\text{H}^+]}{[\text{NAD(P)}^+][\text{glutamate}^{+2-}][\text{H}_2\text{O}]}$$

$$= \frac{[\text{NAD(P)H}]^3}{[\text{NAD(P)}^+][\text{glutamate}^{+2-}] \times 55{\cdot}56 \times \text{antilog pH}} \quad (1)$$

RESULTS

The results of equilibrium measurements with NAD^+ at 27° and various ionic strengths are shown in Table 1. For each separate experiment, the mean value of K_c from measurements of the number of equilibrium mixtures indicated is shown in column (7), and the average value from the several experiments at each ionic strength in column (8). The equilibrium constant is evidently independent of the reactant concentrations and increases with the ionic strength. Analogous results for the $NADP^+$ reaction are recorded in Table 2.

The variation of both equilibrium constants with ionic strength is shown in plots of $16+\log K_c$ against $\sqrt{I}$ (Fig. 1). Below I 0·1, the plots are linear and satisfy an equation of the form:

$$\log K_c = \log K_0 + A\sqrt{I}$$

The slope is 1·4 for NAD^+ and 2·4 for $NADP^+$. The intercepts give values for K_0 at zero ionic strength and 27° of $0{\cdot}64 \times 10^{-15}$ M for NAD^+ and $0{\cdot}193 \times 10^{-15}$ M for $NADP^+$.

Equilibrium measurements were made at four other temperatures from 25° to 38° with I 0·47.

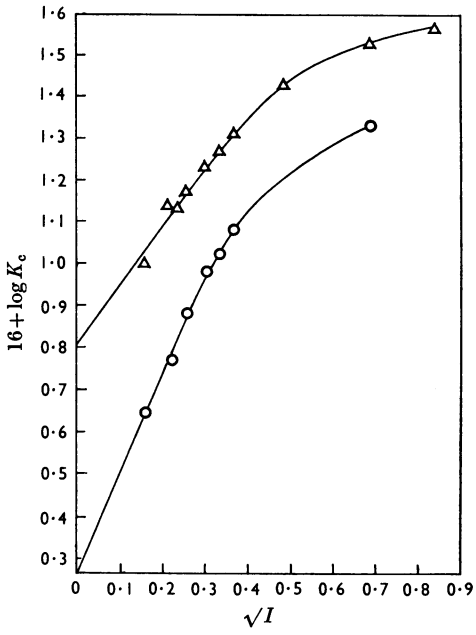

Fig. 1. Effect of ionic strength (I) on the equilibrium constants of the glutamate dehydrogenase reaction with NAD^+ (△) and $NADP^+$ (○) at 27°. The data are from Tables 1 and 2.

Table 2. *Glutamate dehydrogenase equilibrium with $NADP^+$ in phosphate buffers of various ionic strengths at* 27°

Details are as in Table 1.

(1) I	(2) Expt. no.	(3) pH	(4) [$NADP^+$] (μM)	(5) [Glutamate] (mM)	(6) [NADPH] (μM)	(7) $10^{15}K_c$ (M)	(8) $10^{15}K_c$ (av.) (M)
0·026	9	6·93	381	1·35	45	0·443 (3)	0·443
0·051	5	6·90	131	2·0–3·3	36–42	0·586 (5)	0·594
	9	6·94	381	1·35	50	0·601 (3)	
0·067	7	6·96	235	1·34	44	0·756 (3)	0·766
	9	6·93	381	1·35	52–58	0·776 (3)	
0·091	9	6·97	381	1·35	59	0·957 (3)	0·957
0·112	7	6·96	235	1·34	50	1·03 (3)	1·05
	9	6·97	381	1·35	61	1·07 (3)	
0·134	7	6·92	235	1·34	51	1·28 (3)	
	8	6·92	389	1·49	61	1·09 (2)	1·22
	9	6·95	381	1·35	63	1·28 (3)	
0·471	4	6·91	132	2·0–13·3	50–83	2·00 (7)	
	10	6·79	361	2·23	79	2·36 (3)	2·16
	11	6·99	394	2·23	91	2·13 (3)	

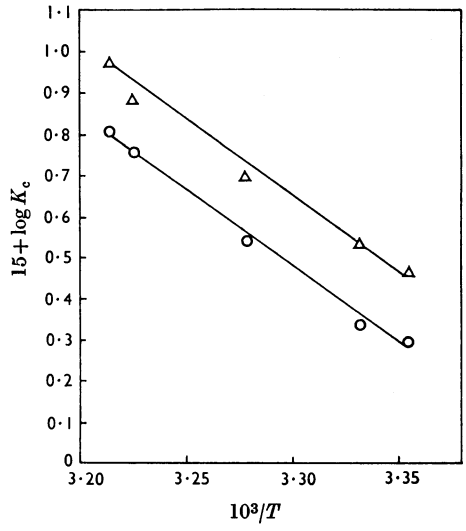

Fig. 2. Effect of temperature on the equilibrium constants of the glutamate dehydrogenase reaction with NAD^+ (△) and $NADP^+$ (○), I 0·47 with respect to phosphate buffer.

Plots of $15+\log K_c$ against $1/T$ (Fig. 2) give values for ΔH of 16·9kcal./mole for the NAD^+ reaction and 17·5kcal./mole for the $NADP^+$ reaction, from the van't Hoff isochore:

$$\mathrm{d}(\log K)/\mathrm{d}(1/T) = \Delta H/2{\cdot}3\boldsymbol{R}$$

The difference between these two values is within the experimental error.

The equilibrium constants were also measured at certain other temperatures and ionic strengths. At 38° and I 0·25, which approximates conditions in the liver (Hohorst, 1960; Williamson *et al.* 1967) the values were $6{\cdot}97\times10^{-15}$M with NAD^+ and $4{\cdot}48\times10^{-15}$M with $NADP^+$. At 25° and I 0·1, the values were $1{\cdot}54\times10^{-15}$M and $0{\cdot}86\times10^{-15}$M respectively.

DISCUSSION

Olsen & Anfinsen (1953) reported values of $2{\cdot}61\times10^{-15}$M and $1{\cdot}78\times10^{-15}$M respectively for the equilibrium constants of the NAD^+ and $NADP^+$ reactions defined by eqn. (1), from direct measurements at 27° and I approx. 0·47. Slightly larger values were obtained under the same conditions in the present work, namely $3{\cdot}40\times10^{-15}$M and $2{\cdot}16\times10^{-15}$M respectively. A possible explanation of these relatively small discrepancies is that coenzyme destruction in the present work was minimized by the use of larger enzyme concentrations to give shorter equilibration times.

The effect of ionic strength on the equilibrium constants has not been studied previously. However, Olsen & Anfinsen (1953) carried out experiments at various ammonium chloride concentrations showing that 2-oxoglutarate and not 2-iminoglutarate is the major product of the reaction. Their results show a distinct upward trend in K_c with increase of ammonium chloride concentration, which is explained by the rather large ionic-strength effect shown here. It might be anticipated that the ionic-strength effect would be greater in the $NADP^+$ system than in the NAD^+ system, because of the additional ionized phosphate group in $NADP^+$. The results (Fig. 1) show that this is the case. The ratio of the equilibrium constant of the NAD^+ system to that of the $NADP^+$ system increases with decrease of ionic strength from 1·57 at I 0·47 to 3·32 at I 0. Olsen & Anfinsen (1953) found the ratio to be 1·47 at I 0·47. Estimates of the standard free-energy change for NADP reduction from this value would clearly be greater than the true value at zero ionic strength.

The equilibrium constant defined by eqn. (1) includes water as a reactant, and the values at 27° obtained by extrapolation to zero ionic strength were $0{\cdot}641\times10^{-15}$M for NAD^+ and $0{\cdot}193\times10^{-15}$M for $NADP^+$. Adopting the usual standard states of the ideal molal solution for dissolved reactants and the pure liquid (55·56M) for water, and correcting to 25° by means of the temperature coefficient determined with I 0·47, the thermodynamic equilibrium constants are:

$$\text{for } NAD^+,\ K_a = 0{\cdot}641\times10^{-15}\times55{\cdot}56\times0{\cdot}83 = 2{\cdot}95\times10^{-14}\text{M}^2$$

$$\text{and for } NADP^+,\ K_a = 0{\cdot}193\times10^{-15}\times55{\cdot}56\times0{\cdot}83 = 0{\cdot}89\times10^{-14}\text{M}^2$$

The temperature correction factor, 0·83, is derived on the assumption that ΔH is 17·0kcal./mole, estimated at I 0·47, and is equal to ΔH_0. Less detailed measurements at lower ionic strengths of 0·056 and 0·10 and only two temperatures gave approximate ΔH values of 16·0 and 18·7kcal./mole respectively.

The standard free-energy changes for the oxidation of glutamate by NAD^+ and $NADP^+$ respectively from:

$$-\Delta G_0 = \boldsymbol{R}T\ln K_a$$

are 18·45 and 19·16kcal./mole. The former value agrees very well with that of 18·28kcal./mole calculated by Burton & Krebs (1953) from thermochemical data for the free energy of formation of glutamate and the standard free energy of reduction of NAD^+, and confirms their view that the apparent discrepancy between their calculated value and that of 17·63kcal./mole derived by Olsen & Anfinsen (1953) from direct equilibrium-constant measurements at high ionic strengths is due to neglect of activity coefficients.

Table 3. *Free-energy data for the glutamate dehydrogenase reactions*

The data refer to aqueous solution at 25°, and are calculated from equilibrium constants measured in phosphate buffers of various concentrations and extrapolated to zero ionic strength.

Reactants	Products	ΔG^0 (kcal./mole)	E'_0 (pH 7) (v)
Glutamate^{+2-} + NAD^+ + H_2O	2-Oxoglutarate^{2-} + NADH + NH_4^+ + H^+	18·45	
Glutamate^{+2-} + $NADP^+$ + H_2O	2-Oxoglutarate^{2-} + NADPH + NH_4^+ + H^+	19·16	
NAD^+ (+ H_2 gas)	NADH + H^+	5·22*	−0·320*
$NADP^+$ (+ H_2 gas)	NADPH + H^+	5·93	−0·335
Glutamate^{+2-} + H_2O	2-Oxoglutarate^{2-} + NH_4^+ (+ H_2 gas)	13·23	−0·126

* From Burton & Wilson (1953).

From equilibrium measurements on the oxidation of propan-2-ol by NAD^+ at low ionic strength, catalysed by yeast alcohol dehydrogenase, together with free-energy data for propan-2-ol and acetone from thermochemical measurements, Burton & Wilson (1953) estimated the standard free energy for the reduction of NAD^+ as 5·22 kcal./mole. Accepting this value, the standard free energy for the reduction of $NADP^+$ can be calculated from the ratio of the thermodynamic equilibrium constants for the glutamate dehydrogenase equilibria:

$$\Delta G_0 = 5{\cdot}22 + \boldsymbol{RT}\ln(2{\cdot}95/0{\cdot}89) = 5{\cdot}93\,\text{kcal./mole}$$

which corresponds to a standard oxidation–reduction potential at pH 7·0 of $E'_0 - 0{\cdot}335$ v. By a similar calculation using the ratio of equilibrium constants estimated by Olsen & Anfinsen (1953) at high ionic strength, Burton & Wilson (1953) obtained ΔG_0 5·44 kcal./mole (E'_0 −0·324 v), but recognized that an error of up to ±0·4 kcal./mole might result from the neglect of activity coefficients.

Free-energy data for the glutamate dehydrogenase reactions are summarized in Table 3.

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